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14. ABSTRACT One of the hallmarks of Parkinson Disease is a deficit in Complex I of the mitochondrial complex chain in the tyrosine hydroxylase (THf)+ neurons in the substantia nigra pars compacta (SNpc) of the brain. This situation allows the dopamine neuron to be put under oxidative stress, thus leaving the DA neuron vulnerable to free radical attack. In attempts to reverse the deficit in Complex I in the cell, in earlier experiments, we used a ketone body, D-beta-hydroxybutyrate (DBHB) in the presence of MPTP to increase oxidative phosphorylation. This change in phosphorylation state afforded protection to the SNpc TH+neurons. One drawback to the use of DBHB is that it is short-acting. In our present experiment, we used a DBHB analogue, glyceryl tris(3-hydroxybutyrate, G3HB) which is the basic DBHB structure that has been altered. Compared to DBHB which increased DBHB levels in plasma by almost 100%, G3HB increased plasma DBHB levels by less than 50%. Increasing the assay sample volume increases brain tissue DBHB levels. In the in vivo studies, the combination of MPTP and G3HB seemed to cause significant mortality in the two strains of mice that we used. This high mortality rate may be strain or breeding house dependent. More studies need to be done to sort this situation out.					
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Report for July 1, 2012 to June 30, 2013

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Introduction

Parkinson's Disease and Mitochondria

PD is the second most common neurodegenerative disease after Alzheimer's Disease and normally manifests itself during the 5th or 6th decade of life. It is characterized by a behavioral phenomenon referred to as TRAP (tremor, rigidity, akinesia and postural instability) which is due mainly, though not exclusively, to the loss of the dopamine neurons in the substantia nigra pars compacta (SNPC) of the brain and their connecting terminals in the corpus striatum (Fahn and Przedborski, 2009). The most widely used treatment for this debilitating disease still remains Levodopa, (L-DOPA) which corrects the symptomology of PD for a while, but does not halt the progression of the disease. Furthermore, this drug has its own set of baggage. PD is essentially (90-95%) a sporadic condition, hence its etiology is not known. However, thus far, we do know that, aside from the death of the DA neurons in the SNpc, 1) there is a greater loss of dopaminergic terminals in the striatum than the loss of dopaminergic neurons in the SNpc (Fahn and Przedborski, 2009); 2) there is an neuroinflammatory component to PD that we and others suspect to be the cause of PD's progressive nature (McGeer et al, 1988; Banati et al, 1998); 3) there is an up-regulation of certain cytokines in the SNpc (Mogi et al, 2000; Mogi et al, 1996; Mogi et al, 1994) 4) the superoxide radical and nitric oxide have been implicated in PD (Hunot et al, 1996) and 5) dysregulation of mitochondria is somehow in play here (Perier et al, 2007). The central hypothesis that encompasses what we know is that following the initiation of the disease, a cascade of deleterious events leading to oxidative injury, macromolecule damage and evoked neuroinflammation all conspire to produce mitochondrial dysfunction and energy failure which ends in the death of the SNpc DA neuron.

Mitochondrial dysfunction and Neurodegeneration. Mitochondria are the powerhouses of the cell. They produce the ATP necessary for neuronal components to engage in reactions geared toward proper function of the neuron. Production of ATP is via the mitochondrial electron transport chain (METC) by the passage of electrons down the chain through a series of complex enzymes as a result of the proton motive force (Huttermann et al, 2008). Proton motive force is initiated by complex I, the first and most complicated of this series of enzymes. Complex I consists of at least 43 subunits, a flavonucleotide, nine iron-sulfur clusters covalently bound lipids and least two ubiquinol binding sites (ref). Its molecular weight is about 900,000 (Keeney et al, 2006). It is one of the main sites at which premature electron leakage of oxygen can occur, thus it is one of main sites for the production of the harmful superoxide radical. When there is a complex I deficiency, such as in PD (Keeney et al, 2006), protons can no longer move down the METC (Keeney et al, 2006), mitochondrial membrane potential collapses and ATP production is compromised (ref). Thus, the neuron is thrown into energy crisis and respiratory failure; the initiating factor for this scenario is still not known. Since the METC is in failure, oxidative phosphorylation cannot proceed, and if oxidative phosphorylation cannot proceed, no ATP can be made. Therefore, a build-up of the superoxide radical occurs, which can lead to increases in other reactive oxygen species (ROS) such as H₂O₂ and the hydroxyl radical, thus the oxidative stress. Many studies have proposed this very scenario for a number of neurodegenerative disorders including PD and AD. There are indicators that this scenario might take place in PD.

Since neuronal mitochondria in PD exhibit a deficit in complex I which can lead to an energy crisis in the neuron, one idea is to stimulate complex I production, which is most likely difficult because the complex I deficit is not limited to the DA neuron. Another avenue is to find an alternate energy source for the brain which we have done in the past (Tieu et al, 2003). D- β -hydroxybutyrate (D β HB) is a ketone body that is produced by both astrocytes and hepatocytes (Guzman et al, 2001). It is also an alternate source of energy in the brain when glucose is lacking such as during starvation (Owen et al, 1967). Studies In a dish, studies have shown D β HB to prevent neuronal damage following glucose starvation. We have shown this same compound to be protective in the MPTP mouse model of PD by enhancing oxidative phosphorylation through complex II (succinate ubiquinone oxidoreductase)

Body of Research

SA I: Compare the effects of D β HB and compounds A and B on mitochondrial function and HDAC activity.

Mitochondria are the powerplants of the neurons and are believed to be at the heart of the oxidative stress in the DA neuron. Here, cellular energy is compromised in situations such as PD as demonstrated by decreases in ATP production, reduction in oxygen consumption, collapse of membrane potential, and excess H₂O₂ production. In an earlier study (Tieu et al, 2003), we used D β HB, a ketone body that is produced by astrocytes (most numerous glia in the brain), as an alternate energy source for brain mitochondria in MPTP-treated mice. Thus, D β HB, infused subcutaneously, enhanced oxidative phosphorylation which led to a reversal of oxidative stress via a mechanism that was dependent on mitochondrial complex II. Since D β HB is a short-acting chemical due to its short half-life, we were asked to compare compounds A and B to the demonstrated protective effects of D β HB in this PD model. These new compounds are altered to increase their half-life. Thus, we are to gauge their effects at the mitochondrial level using the METC (activity of the complexes) and HDAC (histone deacetylase)

SA II .Compare the neuroprotective effects of D β HB and compounds A and B on MPTP-induced neurotoxicity in the SNpc of mice.

Since we have demonstrated that D β HB was neuroprotective in the SNPC of MPTP-treated mice, the second part of this study was to assess the nigrostriatal DA system in the acutely-treated MPTP mouse. The new compounds were to be used in the same manner as D β HB.

Key Accomplishments

The New Compounds

We received one compound, a Glyceryl tris(3-hydroxybutyrate (G3HB), almost an entire year into this award (11 April 2011). The received compound has a molecular weight of 350.36, is about 94% pure and is water soluble. We received a total of 80 grams plus an additional 20 grams in November, 2012. Delays in getting the G3HB to us are unavoidable as this compound had to be synthesized. We never received the second compound. However, in spite of all of the setbacks, we were able to conduct a number of studies.

G3HB in C57 mice: mice received several different concentrations of G3HB via alzet pumps and the MPTP acute dosing schedule (20 mg/kg free base x 4 doses over 8 hours) to determine

whether G3HB offers any protection to SNpc neurons. Mice were then perfused and brains quickly removed, post-fixed overnight in the same fixative, cryoprotected, then frozen and stored at -80°C until used. 30µ sections were collected free-floating and the SNpc was stained for the tyrosine hydroxylase (TH)+ neurons.

Figure 1. Results show that G3HB, at the two highest concentrations, offered some protection to the SNpc TH+ neurons against MPTP.

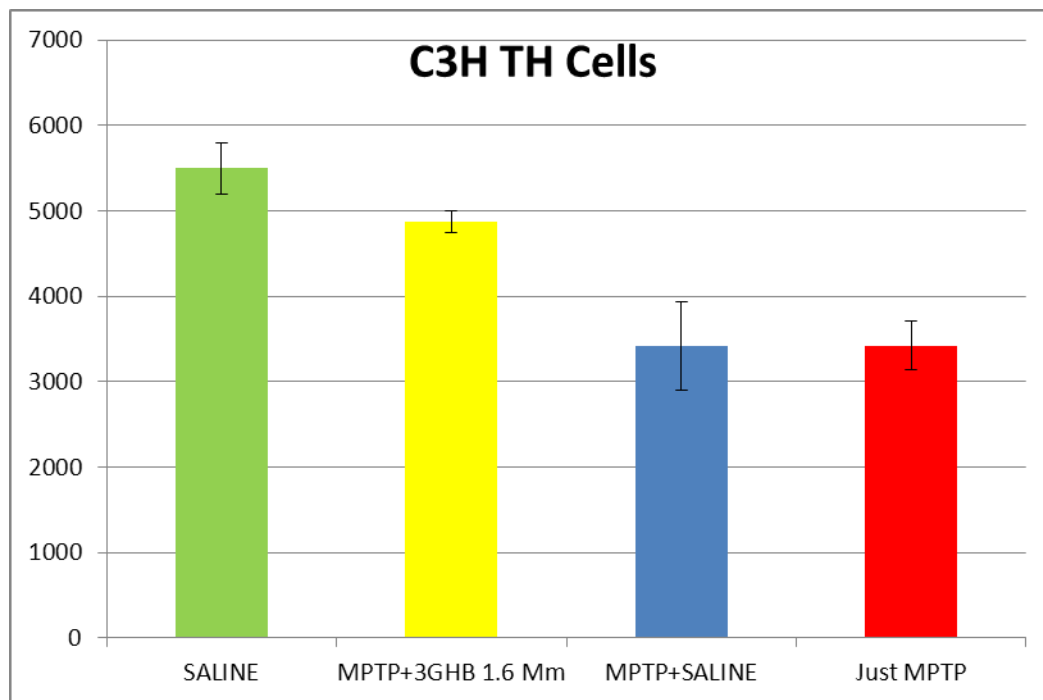
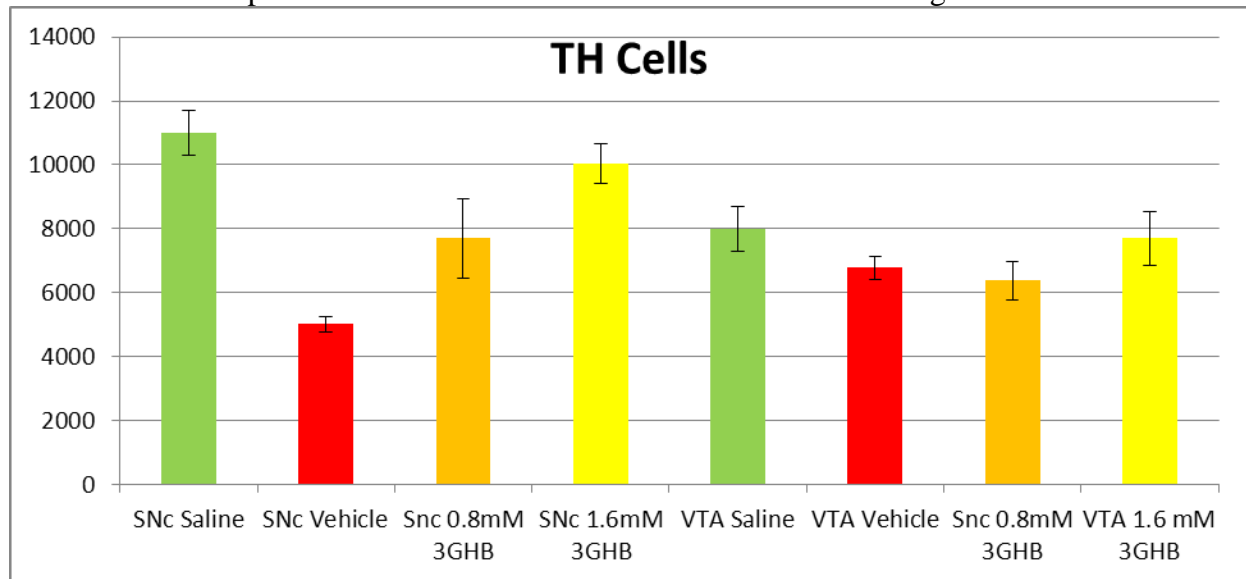


Figure 2. Because of the high mortality rate in the experiments using C57bl/6, we tried the same experiments in the C3H strain of mice. These mice were used because they gave a morphological

and biochemical picture similar to that of the C57bl/6 mice following MPTP administration. As seen in Figure 2, significant protection was afforded to the SNpc DA neurons, but there was a mortality rate of about 50%, when our normal mortality rate in these studies using C57bl/6 mice from the Kingston facility of Charles River is between 5 and 10%.

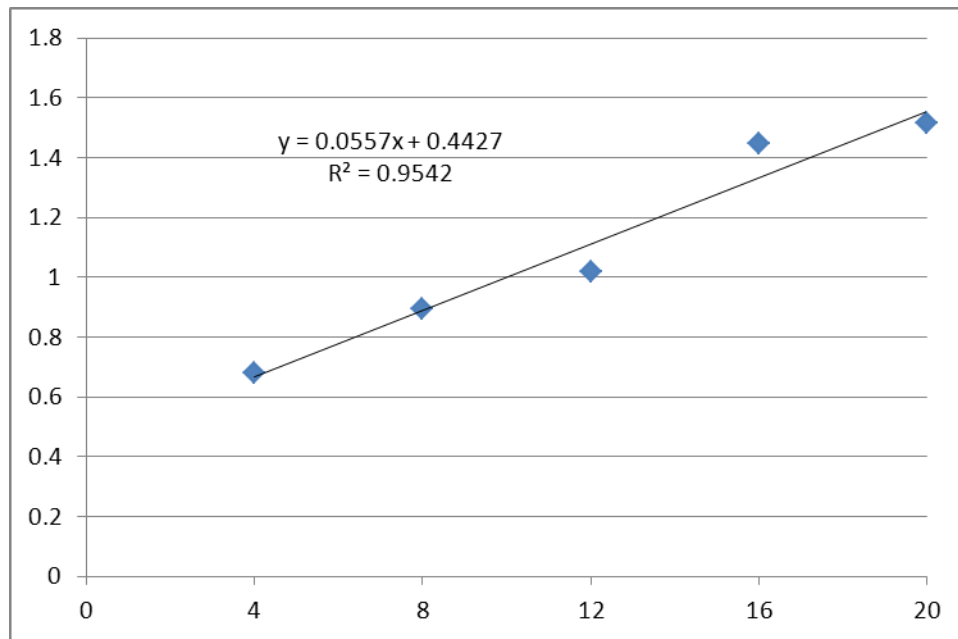


Figure 3. DβHB standard curve. Using the Beta-hydroxybutyrate Assay kit (MAK041) from Sigma Chemical Company from Sigma Chemical Co, we performed standard curves for the measurement of DβHB. Assay parameters and speed and accuracy in pipetting are important for the development of this assay.

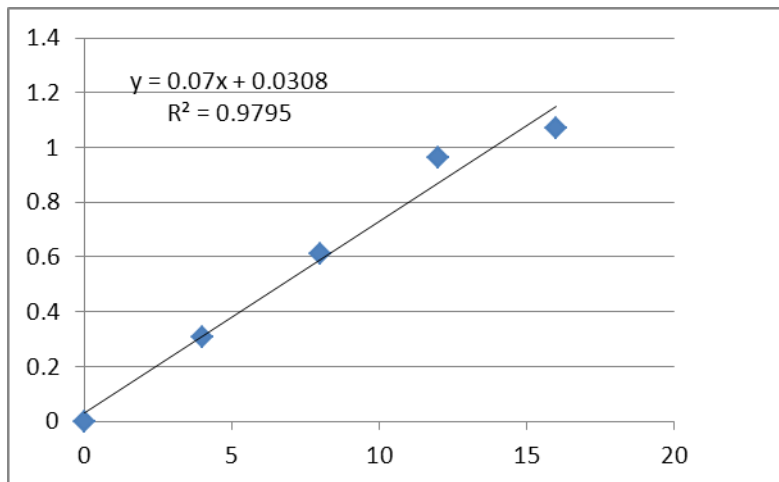


Figure 4a.

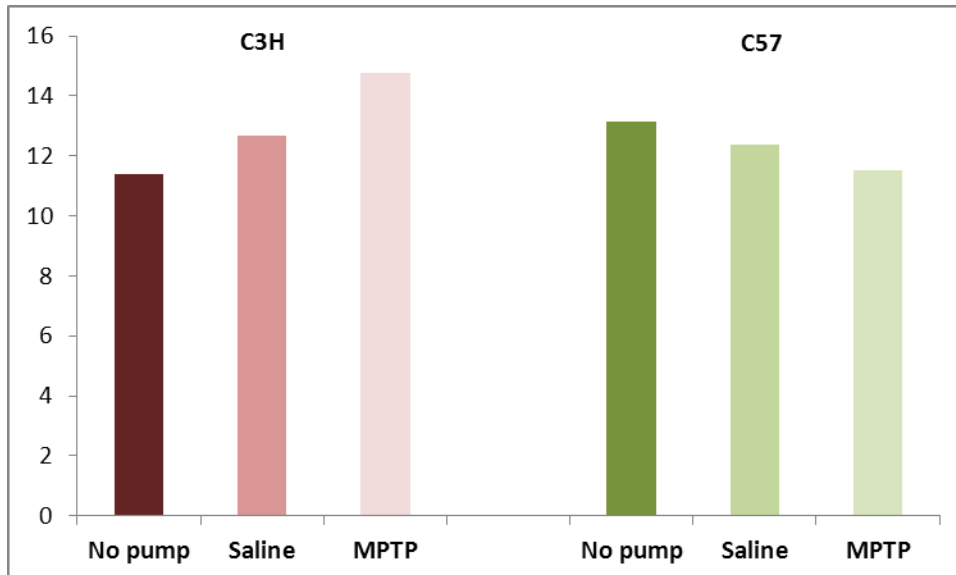


Figure 4b

Figures 4a and 4b represent the results of the assay for tissue DβHB levels. 4a. is the standard curve following the manufacturer's instructions. 4b. shows DβHB brain tissue results in tissues from C57bl/6 and C3H mice that had no pump, pumps delivering saline or pumps delivering saline in animals receiving MPTP. In the C3H group, MPTP injections seemed to cause a significant increase in brain DβHB levels while the same situation in the C57bl/6 mice elicited somewhat of a decrease in brain levels of DβHB. These results need to be clarified.

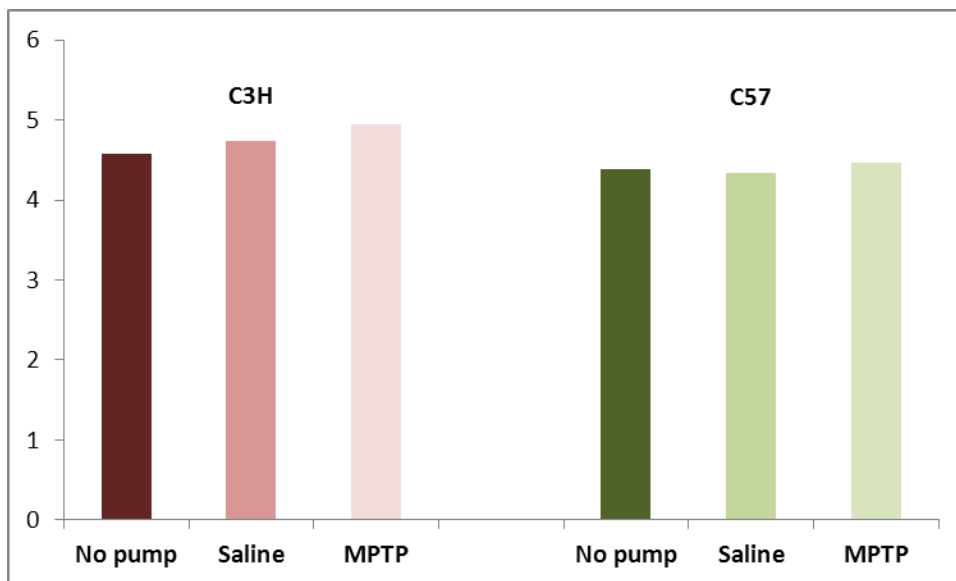
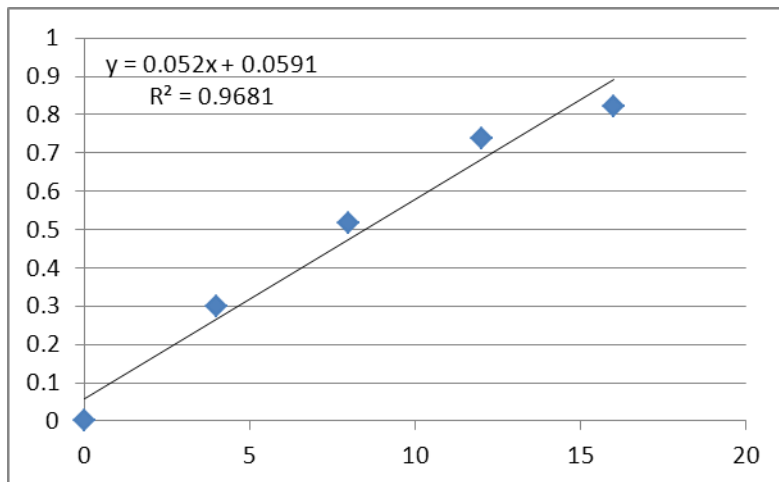


Figure 5.

This figure is a repeat of 4ba and DβHB results are essentially the same in the C3H group as in 4b, although not as pronounced in the MPTP tissues. In the C57bl/6 group, MPTP had no real effect. This experiment attests to the reproducibility of the DβHB assay.

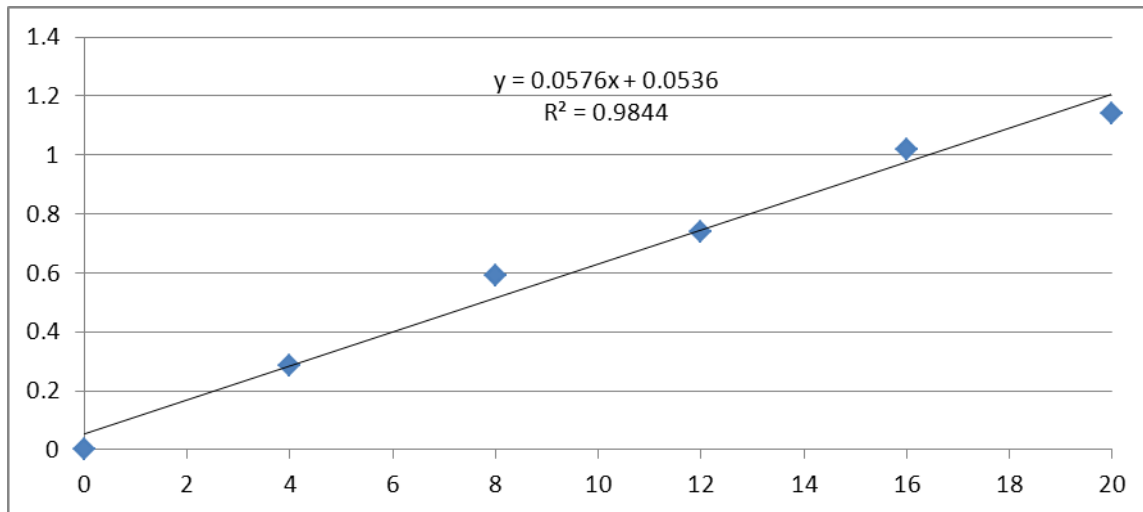


Figure 6a

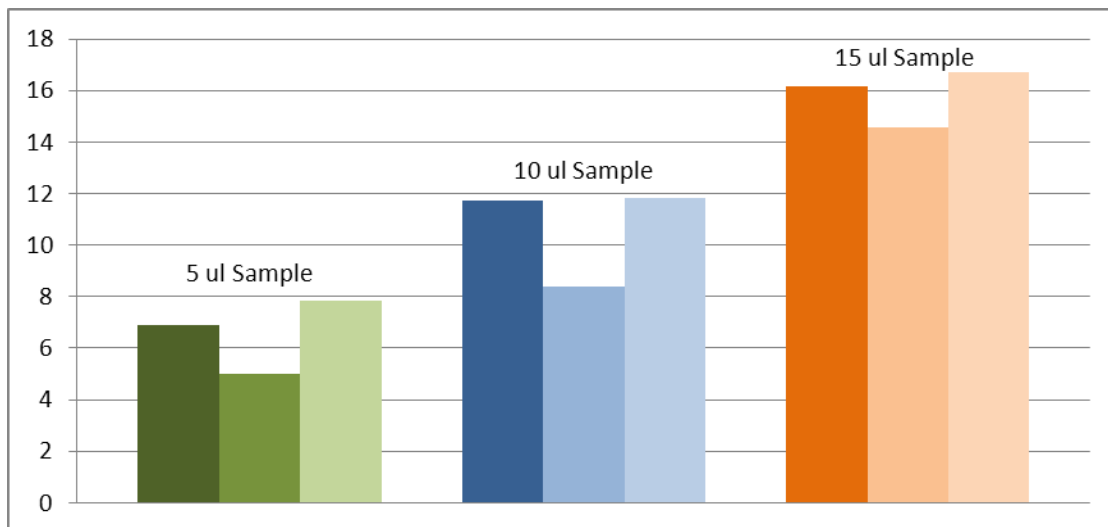


Figure 6b

Figure 6 represents the concentration dependence of the assay for DβHB levels in brain tissue. Here, we increased DβHB concentration by increasing tissue volume. As can be seen, increasing tissue volume increased DβHB tissue levels in a linear fashion.

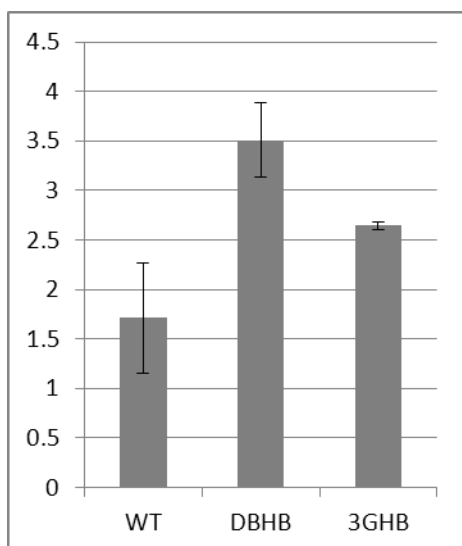
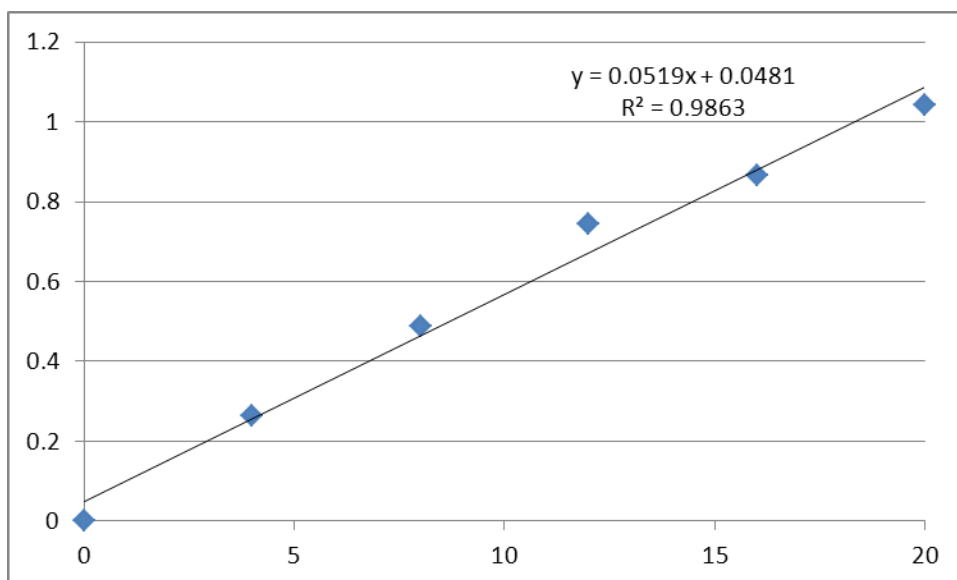


Figure 7. Here, we wanted to see plasma DβHB levels following alzet pump delivery of DβHB and G3HB. C57bl/6 mice were implanted subcutaneously with alzet pumps containing either DβHB or G3HB and euthanized 24 hours later. Blood was collected for plasma levels of DβHB. Noted in Figure 7 is the fact that the mice receiving DβHB had significantly higher levels of plasma DβHB than those animals receiving G3HB.

Reportable Outcomes

We are still trying to work out the dosing of G3HB as this compound does not seem to work well in a pump. We are also concerned about the strains of mice used here.

We can now detect DβHB levels in plasma and in brain tissue.

There was almost a 100% increase in plasma D β HB levels in D β HB pump-implanted mice whereas in G3HB pump-implanted mice, plasma D β HB level increases were less than 50%.

Discussion and Conclusion

One of the hallmarks of PD is a significant decrease in complex I activity that is not necessarily limited to the brain. A major involvement in this progressive disease is the nigrostriatal DA system and its loss of the tyrosine hydroxylase (TH)+ neurons in the SNpc. The DA neuron seems to be under an increased amount of stress at all times which can increase its vulnerability to even subtle changes in the extracellular environment in the SN. Decreases in complex I throws the DA in an oxidative stress such that there is an increased production of the superoxide radical, an up-regulation of the nNos and iNOS enzymes, increased levels of cytokines and chemokines and a neuroinflammation that is cyclic in its pushing the progression of PD. Thus, neuroprotective therapies that are sought, hopefully should halt the progression of PD. One of the deficits that the sought after therapy should reverse is the deficit in complex I activity as lessening this might lessen the oxidative stress on the DA neuron.

In the past, we have used the MPTP mouse model of PD to look into the complex I deficit and its reversal using the ketone body D- β -hydroxybutyrate (D β HB). We found that D β HB can protect the DA neuron in the SNpc (Tieu et al, 2003), not by increasing complex I levels but by enhancing oxidative phosphorylation through a mechanism that is dependent on complex II activity. The one drawback to the use of D β HB as a protective agent in the MPTP mouse model of PD is that it is a short-acting compound. Thus, it was reasoned that extending the half-life of this compound or a similar compound through structural alterations might prove useful. We finally received glyceryl tris(3-hydroxybutyrate, G3HB), an analogue of D β HB that was the result of a structural alteration of the basic compound. We were to replicate our original experiment comparing the G3HB to D β HB.

The G3HB compound is soluble in saline, however we are not so sure that it readily traverses the alzet pump membrane as plasma levels of D β HB in G3HB-infused mice were increased only about 50% compared to the plasma levels of D β HB in D β HB-infused mice. We need to repeat this experiment in a dose-dependent manner. We also need to compare alzet pump versus injection. Furthermore, the mortality rate in the mice was much higher than our usual MPTP mortality rate (5-10%). In this respect, we performed several studies in several strains of mice. Our first test was using an acute MPTP dosing schedule in C57bl/6 mice from Charles River's Kingston facility that were infused with G3HB. To our surprise, all of the mice succumbed. We repeated the experiment and as with the first set, all of the mice succumbed. Since we are researching sensitivity to MPTP among the different strains of mice, we decided to switch strains to the C3H strain as it gave the closest biochemical and morphological picture to that of the C57bl/6 strain. Results in the C3H strain showed 50% mortality in the G3HB plus MPTP groups. We repeated the experiment and received the same results. Our next step here is to use a milder dose of MPTP in G3HB-treated mice. We now want to do this experiment using a subacute MPTP dosing schedule because the acute dosing schedule of MPTP is quite harsh.

Results show that plasma levels of D β HB are increased even in the G3HB-treated groups of mice. Since this is the case, it is our opinion that maybe we need a different approach in terms of the use of this compound and that may be injection rather than alzet pump.

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